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Simultaneous Presence of Different *Borrelia burgdorferi* Genospecies in Biological Fluids of Lyme Disease Patients

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Oligonucleotide primers based on *Borrelia burgdorferi* sensu lato *ospA* gene sequences have been designed for use in the PCR to type all (SL primers) or each (GI to GIII primers) of the *B. burgdorferi* sensu lato genospecies involved in Lyme disease. These genospecies-specific primers were then used in the PCR on 24 biological fluids collected from 18 neuroborreliosis patients. Among the samples tested, 20 contained DNA from *Borrelia garinii*, 11 contained DNA from *B. burgdorferi* sensu stricto, and 10 contained DNA from *Borrelia afzelii*. In toto, 10 patients appeared to have been infected by a single genospecies and 8 were infected by more than one Lyme disease-associated genospecies. Serum specimens from six patients were absorbed with heterologous antigens and tested by Western blotting (immunoblotting). In four cases, residual immunodetection revealed specific epitopes of genospecies also detected by PCR; in two of them, the concordant results indicated pluri-infection of the patients. In the other two cases, Western blotting showed specific antibodies for two genospecies of *Borrelia*, while PCR detected DNA from only one. In summary, the data underscored the relatively high prevalence of pluri-infections in Lyme disease and confirmed the association of *B. garinii* with neuroborreliosis.

Lyme disease is a tick-borne multisystemic illness distributed throughout the Northern Hemisphere and caused by a spirochetal species called *Borrelia burgdorferi* sensu lato (6, 13). The clinical symptoms of Lyme disease in humans vary from an acute skin rash (erythema chronicum migrans) to severe arthritic, neurologic, dermatologic, and cardiac manifestations, with a predominance of neurologic and dermatologic symptoms in Europe and of arthritis in the United States (21).

Significant divergence among isolates of *B. burgdorferi* sensu lato was first suggested by differences in monoclonal antibody reactivity, plasmid profiles, and the clinical manifestations of Lyme borreliosis in Europe versus North America (3, 14, 22). Recent studies using 16S rRNA and flagellin gene sequence analysis, DNA-DNA hybridization, multilocus enzyme electrophoresis, plasmid profiles, immunoblotting, and reactivity with monoclonal antibodies have demonstrated that *B. burgdorferi* sensu lato can be divided into three genospecies, i.e., *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (7, 26, 27, 31–37). It seems that specific genospecies are involved in distinct clinical manifestations of the disease; indeed, infections by *B. burgdorferi* sensu stricto tend to lead to arthritic symptoms, while those by *B. garinii* and *B. afzelii* appear to cause neurological complications and cutaneous manifestations, respectively (1, 2, 7). A fourth genospecies, *Borrelia japonica*, has recently been discovered in Japan (38). It is considered to be a *B. burgdorferi* sensu lato genospecies because of its protein and genetic similarities with the three other genospecies but actually does not seem to be involved in Lyme disease.

Six major outer surface proteins, OspA, OspB, OspC, OspD, OspE, and OspF, in Lyme disease spirochetes have been described (16, 24, 37). The *ospA* and *ospB* genes, located on the linear 49-kb plasmid present in all isolates belonging to the three genospecies, have been cloned and sequenced (14, 27). The corresponding proteins, OspA and OspB, have been found to vary in molecular weight, in reactivity with various monoclonal antibodies, and in gene sequence (7, 22), depending on the genospecies. However, their functional importance remains unclear. Studies have indicated that OspA and OspB proteins may play a role in the adherence of *B. burgdorferi* sensu lato to different eukaryotic cells (8) and in its invasion of and movement through the cells (29). It seems also that OspA and OspB proteins possess B-cell-mitogenic and cytokine-stimulatory properties (19). These important findings concerning a possible function of the OspA protein in the virulence of *B. burgdorferi* sensu lato, the high degree of stability of the linear plasmid carrying *ospA* and *ospB* during in vitro cultivation (30), and the effectiveness of OspA protein in eliciting protective immunity (12) prompted us to develop a molecular assay capable of targeting genospecies-specific *ospA* sequences. Conventional immunological methods (enzyme-linked immunosorbent assay [ELISA] and indirect immunofluorescence assay) (5) currently used for diagnosis are not capable of distinguishing the three genospecies involved in Lyme disease. These tests also appear to yield some false-positive and false-negative results and to lack sensitivity. In the present work, we have derived from *ospA* gene sequences several primer sets for the PCR allowing the detection of *Borrelia* species associated with Lyme disease and the differentiation among genospecies, i.e., *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*. These primers were used on cultured bacteria and on clinical specimens obtained from neuroborreliosis patients. PCR data showed that most patients with neurological symptoms have been infected by *B. garinii* and that several of them carried DNA from more than one genospecies in their biological fluids. Independent evidence for pluri-infection was also obtained by a Western blot (immunoblot) approach, which detected

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antibodies corresponding to specific epitopes of more than one genospecies in some clinical samples tested.

MATERIALS AND METHODS

***Borrelia* strains and culture.** *Borrelia* cultures were maintained in BSKII medium in 50-ml tubes at 37°C (4). The growth was checked every 2 days by dark-field microscopy, and the bacteria were subcultured twice a week. Among the 23 strains used in this study, 14 came from ticks: 4 from *Ixodes persulcatus* (Hp3 from Japan, Ip21 and Ip90 from Russia, and Iper3 from Russia), 1 from *Ixodes dammini* (B31 from the United States), and 9 from *Ixodes ricinus* (G25 from Sweden; IRS, Ne11H, and VS461 from Switzerland; MK-5 and MK-6 from Hungary; and N34, ZQ1, and ZS7 from Germany). Among nine others, five came from cerebrospinal fluid (297 from the United States; IP1, IP2, and IP3 from France; and P/Bi from Germany) and four were from skin biopsies (AcaI and UM01 from Sweden and P/Gau and pKo from Germany).

DNA isolation from *Borrelia* strains and preparation of clinical specimens for PCR amplification. Total DNA from all *Borrelia* strains was extracted as described previously (14, 27). DNA concentrations were determined spectrophotometrically by measuring the A_{260} (20). For DNA amplifications, 100 ng of the preparations was used as template DNA.

Samples (3 ml) of biological fluids (urine, serum, cerebrospinal fluid, or synovial fluid) were centrifuged for 10 min at 12,000 × *g* to pellet spirochetes. The pellets were washed twice in phosphate-buffered saline (PBS) (140 mM NaCl, 2.68 mM KCl, 8.09 mM $Na_2HPO_4 \cdot 2H_2O$, 1.47 mM KH_2PO_4 [pH 7.2]), resuspended in 30 μl of distilled water, and boiled for 10 min. Samples of 10 μl were removed and processed for PCR amplification.

DNA amplification. Primers were synthesized on an Applied Biosystems, Inc., DNA synthesizer. Primer sequences are listed below (see Table 1). PCRs were performed with a Techne PH-C1 (New Brunswick Scientific) thermocycler. *Taq* DNA polymerase, the four deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), and the reaction buffer were used as recommended by the supplier (Boehringer Mannheim Biochemica, Mannheim, Germany). A 20-pmol sample of each primer was used per 50-μl reaction mixture. A 100-ng sample of total genomic DNA was amplified for 35 cycles under the following conditions: 93°C, 1 min; 69°C, 1 min; and 72°C, 1 min. For the GIII primer set, the annealing step was performed at 68°C for 1 min. For the SL primer set, the annealing step was performed at 65°C for 1 min. Before amplification, samples were overlaid with 50 μl of mineral oil (Sigma). Where indicated, amplification primers (c/c′ primers) targeting the chromosome of *B. burgdorferi* sensu lato were used (28).

PCR amplification products were resolved onto 2.0% agarose electrophoresis gels and visualized under UV light with ethidium bromide.

In addition, assays were performed on biological fluids from healthy individuals (serum, urine, cerebrospinal fluid, and synovial fluid) which were uninfected or mock infected with 10^2 spirochetes of strain ZS7 in order to check that the amplification reaction proceeded correctly under these conditions. The results indicated that samples mixed with *B. burgdorferi* ZS7 cells were positive in the assay, confirming that the processing of these biological fluids was appropriate (data not shown). To test the presence of inhibitory substances and to provide a positive control in the PCR assay, amplifications were also performed with primers targeting the APOE-C1-C2 gene cluster on human chromosome 19 (9). Physical containment measures ensured the absence of DNA contamination in the PCR procedure, yielding negative results among the suspected cases tested routinely.

Computer analysis and nucleotide sequence determination. Sequence alignments and the construction of the phylogenetic tree were done on a DECstation 5000/125 computer with the Pileup facility of the Genetics Computer Group sequence analysis software package (version 7.2, 1992; Genetics Computer Group, University Research Park, Madison, Wis.). The following parameter values were used: gap weight, 5.0; gap length weight, 0.3.

The nucleotide sequences of PCR-amplified fragments were determined by the dideoxy chain termination technique with the Prism ready reaction dye deoxy-terminator cycle sequencing kit (Applied Biosystems).

The *ospA* sequence of *Borrelia* strain 297 was kindly provided by Joe Frantz (Pfizer, Lincoln, Nebr.). The *ospA* sequences of *Borrelia* strains AcaI and Ip90 have been published elsewhere (14).

Nucleotide sequence accession numbers. The *ospA* sequences have been assigned accession no. X16467, X14407, M57248, X60300, S48323, M88764, X66065, and S48322 for *Borrelia* strains ZS7, B31, N40, Goë2, P/Bi, B29, ZQ1, and PKO, respectively. The *ospA* sequences of G25 and VS461, obtained in our laboratory (to be published elsewhere), have been entered in the EMBL/GenBank databases with accession numbers Z29086 and Z29087, respectively.

Patients, sera, and biological fluids. Sera used in this study were from Lyme disease patients diagnosed at the Cliniques Universitaires St. Luc (Brussels, Belgium) and at the Ste. Thérèse hospital (Bastogne, Belgium) by ELISA with *B. burgdorferi* sensu stricto B31 flagellin antigen and confirmed with titration by indirect immunofluorescence assay using immunoglobulin G (IgG) or IgM and whole bacterial cells or by PCR using the primers of Rosa and Schwan (28) which are able to detect all *Borrelia* sensu lato strains involved in Lyme disease. All patients had a detailed clinical history of the disease with neurological symptoms, including radiculoneuritis, paresthesia, and facial paralysis (monoplegia, 14 patients; diplegia, 4 patients). All clinical specimens were collected from patients at the time of diagnosis, before antibiotic treatment, except for patient 10, who underwent antibiotic treatment a few days before sampling.

Protein samples and Western blotting. Spirochetes from 40-ml cultures were harvested at maximal densities by centrifugation at 10,000 × *g* for 30 min and washed twice in PBS (pH 7.2). The final pellets were suspended in 500 ml of TE buffer (10 mM Tris [pH 7.6], 1 mM EDTA, 10 mM phenylmethylsulfonyl fluoride) and sonicated for 4 min in an ice bath with a 500-W ultrasonic processor (Vibra cell from Sonics and Materials, Inc., Danbury, Conn.). The protein contents were determined by the method of Lowry et al. (18). The resulting suspensions were adjusted with PBS to give a final protein concentration of 2 mg/ml and stored at −20°C until use.

Total protein lysates of *B. burgdorferi* sensu lato were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide) by the method of Laemmli (15). Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Bedford, Mass.) and subjected to immunological detection as described previously (10). Human sera were diluted 1:100. Reference samples of transblotted total proteins and molecular weight protein markers were visualized by an AuroDye forte staining (RNP490; Amersham International, Amersham, United Kingdom). Polyvinylidene difluoride membranes were photographed under UV light (11).

Antibody absorption. Sera were absorbed with lysates of three strains of *Borrelia*: B31, N34, and UM01, each representative of one genospecies. One milliliter of each diluted serum (1:50) containing 2 mg of proteins of total bacterial extracts was added to an equal volume of Tris-buffered saline (twofold concentrated) with 0.1% (vol/vol) Tween 20, 2% (wt/vol) milk powder, and 10 mM phenylmethylsulfonyl fluoride, and the mixture was incubated for 3 h at 37°C before being centrifuged. The antibody absorption by a given antigen extract was checked by Western blotting, showing the absence of any remaining antibodies against the absorbing agent. In parallel experiments, in which sera had been incubated with pools of different strains belonging to each genospecies, remaining antibodies were identical to the ones revealed by sera absorbed with only one of these strains (data not shown), stressing the validity of the absorption procedure in diagnosing the infecting genospecies.

RESULTS

Selection of primers and analysis of the sensitivity and specificity of the PCR amplifications. In order to select PCR primers suitable for amplification of all *B. burgdorferi* sensu lato isolates and for differentiation of the three genospecies involved in Lyme disease, we have aligned and compared *ospA* sequences from 13 Lyme spirochete isolates. A phylogenetic tree was constructed on the basis of the *ospA* nucleotide sequences (Fig. 1). It revealed the presence of three *ospA* groups, which were named GI to GIII. They coincided with the three genospecies of *B. burgdorferi* sensu lato previously described (3, 7). The GI, GII, and GIII isolates corresponded to *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, respectively.

The SL primers were chosen to amplify the DNA of all Lyme disease isolates. The GI, GII, and GIII primers, named to match the corresponding groups, were aimed at targeting specific *ospA* sequences of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, respectively. The nucleotide sequences of the primers, their expected specificities, and their positions on the *ospA* DNA sequence are described in Table 1. All primers were run on 23 *B. burgdorferi* sensu lato strains and on 3 unrelated *Borrelia* species, i.e., *B. hermsii*, *B. parkeri*, and *B. turicatae* (data not shown).

The SL primers were able to amplify the expected specific DNA fragment with all *B. burgdorferi* sensu lato strains used in this study (Fig. 2B). Furthermore, the seven *B. burgdorferi* sensu stricto isolates (297, B31, IP1, IP2, IP3, IRS, and ZS7) tested were positive in the PCR assay using the GI primers and were negative when the GII and GIII primers were used (Fig. 2A, lanes 1). GII primers were able to amplify *ospA* DNA from the nine tested *B. garinii* isolates (G25, Hp3, Ip90, MK-5, MK-6, N34, Ne11H, P/Bi, and ZQ1), but amplification of these DNA samples was obtained with neither the GI nor the GIII genospecies-specific primers (Fig. 2A, lanes 2). Finally, the seven *B. afzelii* isolates (AcaI, Ip21, Iper3, P/Gau, PKo, Uo1, and VS461) used in this study were positive in the PCR using the GIII genospecies-specific primers but were negative in the

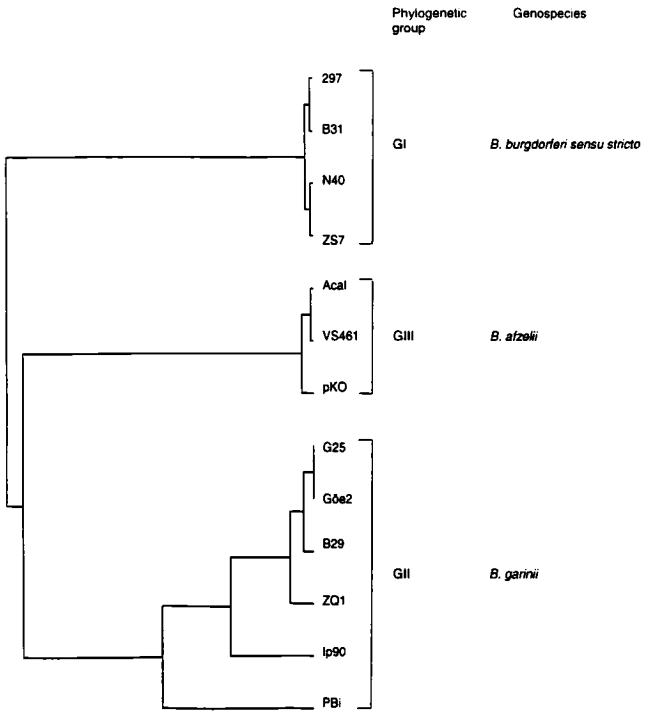

FIG. 1. Phylogenetic tree of the *ospA* DNA sequences derived from 13 Lyme disease isolates. The phylogenetic groups and corresponding genospecies are shown.

PCR using the other two genospecies-specific primers (Fig. 2A, lanes 3). As expected, none of the primer sets was able to amplify DNA from *B. hermsii*, *B. parkeri*, or *B. turicatae* (data not shown).

The detection threshold of the PCR assay was determined by performing amplification reactions with a controlled number of cells. A stock sample of *B. burgdorferi* sensu stricto isolate ZS7 was serially diluted in PBS. The number of *Borrelia* cells ($\pm$ 2.0 $\times$ 10^7 cells per ml) in the stock sample was estimated, and aliquots of the dilution were tested for amplification using the SL primers. It was found that a template DNA input corresponding to about five spirochetes was sufficient to detect the amplified fragment in 10% of the reaction volume (data not shown). As expected, no signal was observed when template DNA was omitted from the amplification reaction mixture.

The specificity of the amplification reactions was confirmed by enzyme restriction analysis. The SL and genospecies-specific primer-amplified DNA fragments were digested with restriction enzymes and analyzed on agarose gels. In all cases, the sizes of the restriction fragments were consistent with the expectations. Examples of the data are shown in Fig. 3C and D; it can be seen that the SL and GII primer-amplified DNAs generated the expected restriction fragments.

PCR analysis of clinical samples with genospecies-specific primers. A total of 24 clinical specimens (12 serum specimens, 10 urine specimens, and 2 cerebrospinal fluid specimens) collected from 18 neuroborreliosis patients (see "Patients, sera, and biological fluids" in Materials and Methods) were subjected to PCR analysis using various sets of oligonucleotide primers. These include the c/c′ primers of Rosa and Schwan (28), which target the chromosomal DNA of *B. burgdorferi* sensu lato; the SL primers; and the three genospecies-specific primers based on *ospA* gene sequences. As seen in Table 2, all samples were positive in PCR amplifications with both c/c′ and SL primers, confirming the diagnosis of Lyme disease. In addition, GI primers, specific for *B. burgdorferi* sensu stricto, detected spirochetal DNA in 11 samples; GII primers, which are specific for *B. garinii*, detected spirochetal DNA in 20 samples; and GIII primers, which target *B. afzelii* DNA, detected spirochetal DNA in 10 specimens.

Thus, 14 patients of the 18 tested appeared to have been infected by *B. garinii*, 9 appeared to have been infected by *B. burgdorferi* sensu stricto, and 7 appeared to have been infected by *B. afzelii*. Indeed, four patients carried genetic material from two genospecies, and four others carried DNA from the three known Lyme disease-associated genospecies. Representative results (for patient 3) are shown in Fig. 4b. In this case, it can be seen that the SL primers and two genospecies-specific primers (GII and GIII) were able to amplify the specific DNA fragment indicating that this patient was infected by *B. garinii* and *B. afzelii* genospecies. In order to exclude the possibility of artifact contamination, the specificity of DNA amplification was confirmed by restriction analysis (Fig. 3B) for the eight samples in which DNAs of multiple strains had been detected. DNAs amplified by GII primers from patients 15 (pluri-infection) and 18 (monoinfection) were also sequenced and compared with *ospA* DNA from reference strain ZQ1 (*B. garinii*) to rule out contamination of samples by *B. burgdorferi* sensu lato strains used as controls in the PCR procedure. The comparison showed that amplified *ospA* sequences from both patients 15 and 18 diverged from that of the control ZQ1 strain and also from *ospA* sequences derived from several known *B. garinii* isolates (14) (data not shown). These data indicated that arti-

TABLE 1. PCR primers for detection and classification of *B. burgdorferi* sensu lato genospecies

Primer set	Oligonucleotide sequence	Target species	Length (bp)	Position and orientation on DNA sequence[a]
SL	5′-AATAGGTCTAATAATAGCCTTAATAGC-3′	*B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*	27	21→47
	5′-CTAGTGTTTTGCCATCTTCTTTGAAAA-3′		27	302←328
GI	5′-AACAAAGACGGCAAGTACGATCTAATT-3′	*B. burgdorferi* sensu stricto	27	139→165
	5′-TTACAGTAATTGTTAAAGTTGAAGTGCC-3′		28	655←682
GII	5′-TGATAAAAAC AACGGTTCTG GAAC-3′	*B. garinii*	24	201→224
	5′-GTAACTTTCAATGTTGTTTTGCCG-3′		24	522←545
GIII	5′-TAAAGACAAAACATCAACAGATGAAATG-3′	*B. afzelii*	28	347→374
	5′-TTCCAATGTTACTTTATCATTAGCTACTT-3′		29	508←536

[a] All positions correspond to the numbering of the *B. burgdorferi* sensu stricto B31 *ospA* sequence.

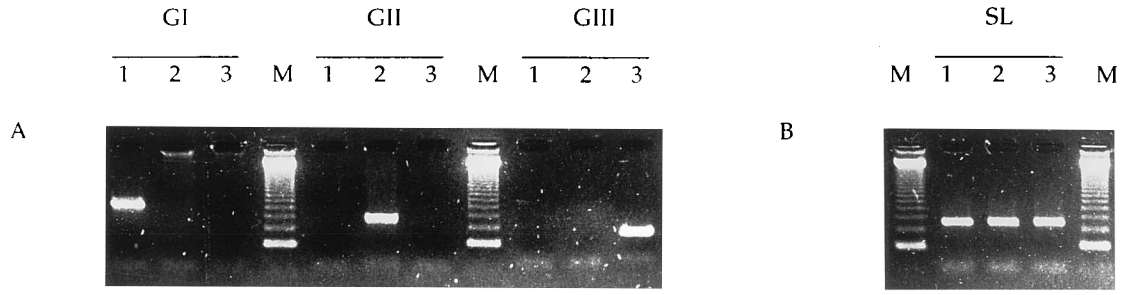

FIG. 2. Amplification specificities of the GI, GII, and GIII (A) and SL (B) primer sets with three representative Lyme disease isolates belonging to each of the three genospecies: *B. burgdorferi* sensu stricto B31 (lanes 1), *B. garinii* ZQ1 (lanes 2), and *B. afzelii* VS461 (lanes 3). A 10% portion of the reaction volume was analyzed on a 2.0% agarose electrophoresis gel. Lanes M, 123-bp DNA ladder (Life Technologies).

fact contamination of the samples did not occur, and they underscored the extensive heterogeneity of *B. burgdorferi* sensu lato strains in nature.

Western blot analysis of sera absorbed with heterologous *Borrelia* antigens. Independent evidence for pluri-infection of patients suffering from neuroborreliosis was searched for by using an immunological approach. The method consisted of probing recognition of species-specific epitopes, using sera from infected individuals which were absorbed separately with total proteins derived from each of the three *B. burgdorferi* sensu lato genospecies. By this procedure, antibodies specific for an infecting strain(s) were expected to be maintained in sera previously absorbed with proteins of genospecies not involved in the infection. On the other hand, they should be eliminated by absorption with proteins homologous to the infecting strain(s). Thus, the probing of the absorbed sera on Western blots carrying proteins of the three genospecies should lead to the identification of species-specific epitopes. Hence, a case of monoinfection would allow the detection of specific epitopes from only one genospecies, whereas in the case of pluri-infections, polypeptides specific for more than one genospecies would be detected.

In this study, 6 serum specimens (from patients 1 to 6) were selected from the 24 samples studied by PCR (four of them contained DNA from a single genospecies, while two others contained DNA from two or three genospecies [Table 2]).

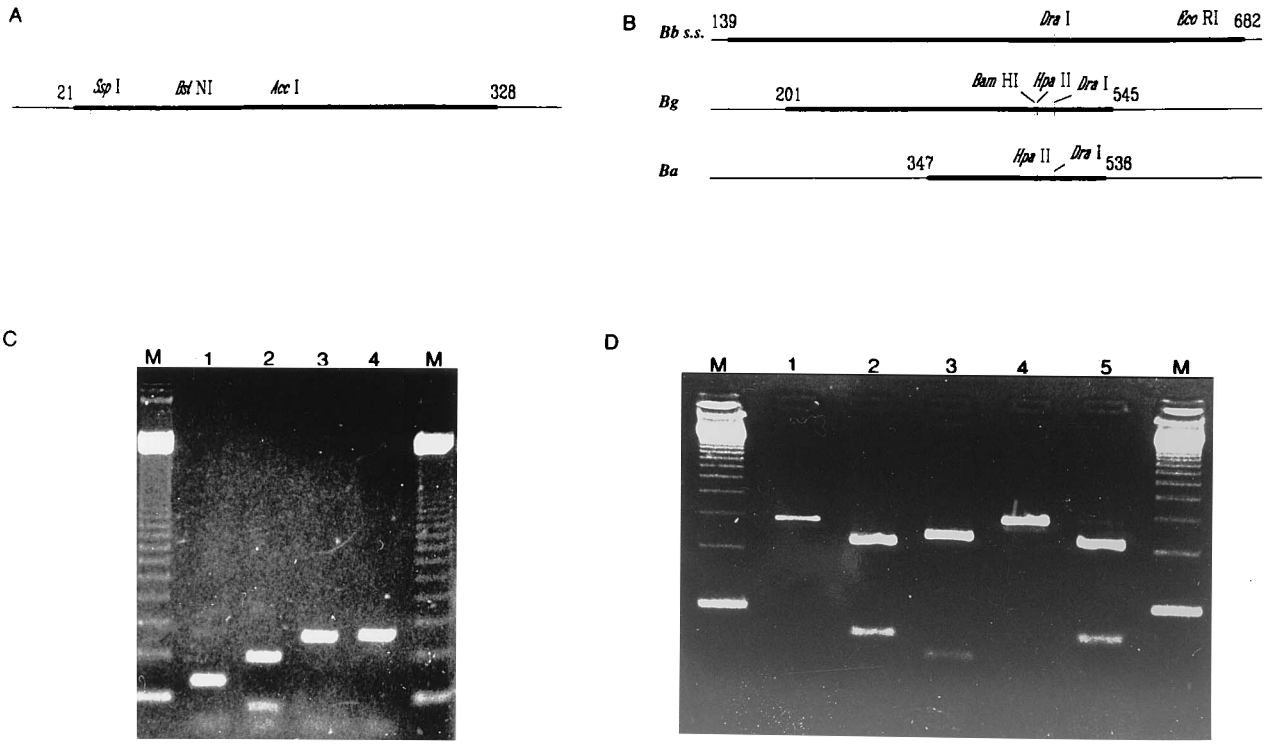

FIG. 3. (A and B) Partial physical maps of *ospA* genes from *B. burgdorferi* sensu lato (A) and from *B. burgdorferi* sensu stricto (*Bb s.s.*), *B. garinii* (*Bg*), and *B. afzelii* (*Ba*) genospecies (B). Numbers refer to nucleotide positions in the *ospA* sequences. Relevant restriction enzymes for identification (A) and for classification (B) are indicated. The amplified DNA fragments (thick lines) are indicated. (C and D) Restriction analysis of the *B. garinii* ZQ1 DNA amplified by the SL and the GII primer sets, respectively. (C) A 10% portion of the PCR volume was either digested with *Acc*I (lane 1), *Bst*NI (lane 2), or *Ssp*I (lane 3) or left undigested (lane 4). (D) A 10% portion of the PCR volume was either left undigested (lane 1) or digested with *Bam*HI (lane 2), *Dra*I (lane 3), *Eco*RI (lane 4), or *Hpa*II (lane 5). The restricted DNA fragments were separated on a 2.0% agarose gel. Lanes M contain a 123-bp DNA ladder (Life Technologies). Expected sizes of the restricted DNA fragments (in base pairs): *Acc*I, 155 and 153, *Bst*NI, 84 and 224; *Ssp*I, 12 and 296; *Bam*HI, 260 and 85; *Dra*I, 285 and 60; *Eco*RI, 545; and *Hpa*II, 264 and 81.

TABLE 2. PCR of clinical samples with genospecies-specific primers

Patient	Specimen source(s)	Result with primer(s)[a]:			
		c/c′ and SL	GI	GII	GIII
1	Serum	+	+	−	−
2	Urine	+	−	−	+
3	Urine and serum	+	−	+	+
4	Serum	+	+	+	+
5	Serum	+	+	−	−
6	Serum	+	+	−	−
7	Serum	+	+	+	−
8	Urine	+	−	+	−
9	Urine	+	−	+	−
10	Serum	+	−	+	−
11	Urine	+	−	+	−
12	Urine and serum	+	−	+	+
13	Urine and serum	+	+	+	−
14	Urine and serum	+	−	+	−
15	Urine and serum	+	+	+	+
16	Serum	+	+	+	+
17	CSF[b]	+	+	+	+
18	Urine and CSF	+	−	+	−

[a] c/c′ primers (28) recognize chromosomal DNA; SL primers recognize *ospA* sequences of all *B. burgdorferi* sensu lato strains; and primers GI, GII, and GIII recognize *ospA* sequences specific for *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, respectively. +, amplification; −, no amplification.

[b] CSF, cerebrospinal fluid.

Representative results are shown in Fig. 4 for the serum of patient 3. As seen in Fig. 4a, lanes A1, B1, and C1, antibodies in the nonabsorbed serum recognized proteins of the three genospecies involved in Lyme disease: *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*. This serum, recognizing a limited number of proteins, was chosen for the sake of an easier understanding of the absorption patterns. Upon separate absorption of the serum with proteins of each genospecies, residual antibody reactivity on the blots was checked. Lanes A2, B3, and C4 in Fig. 4a revealed that no polypeptides corresponding to the absorbing genospecies were detected anymore, indicating complete absorption. On the other hand, serum absorbed with *B. burgdorferi* sensu stricto proteins detected specific epitopes of both *B. garinii* (Fig. 4a, lane B2) and *B. afzelii* (Fig. 4a, lane C2). Furthermore, serum absorbed with *B. garinii* proteins did not react with proteins of *B. burgdorferi* sensu stricto (Fig. 4a, lane A3) but identified specific epitopes of *B. afzelii* (Fig. 4a, lane C3). Finally, serum absorbed with *B. afzelii* proteins did not recognize *B. burgdorferi* sensu stricto proteins (Fig. 4a, lane A4) but revealed a polypeptide specific to *B. garinii* (Fig. 4a, lane B4). Taken together, these results indicated that the serum of patient 3 contained antibodies against two distinct *Borrelia* sensu lato genospecies, i.e., *B. garinii* and *B. afzelii*, in agreement with PCR data (Fig. 4b) which identified DNA of these two genospecies in both the urine and the serum of the patient. Results obtained with five other serum specimens are summarized in Table 3. It can be seen that the sera of patients 1 and 2 contained antibodies specific for a single genospecies, *B. burgdorferi* sensu stricto and *B. afzelii*, respectively, and that the serum of patient 4 reacted with specific epitopes from each of the three genospecies. These results agreed with the PCR data (Fig. 4b). However, in two cases (patients 5 and 6), the Western blot method was at variance with the PCR approach, since it detected specific antibodies against two genospecies, whereas PCR was able to identify only one of them.

DISCUSSION

Our aim was to design *ospA*-based primers for the PCR allowing the detection of all *Borrelia* spp. associated with Lyme disease and differentiation between the genospecies, i.e., *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*. Recently, Rosa and coworkers (27) have designed four PCR primer sets based on *ospA* sequences. Three of them were specific to *B. burgdorferi* sensu stricto or *B. garinii* genospecies, and the last one identified all *B. burgdorferi* sensu lato strains irrespectively of their origin. However, these primers were not able to target directly the *B. afzelii* genospecies and thus were not suitable for detection of *B. afzelii* strains in multiply infected clinical specimens. Using genospecies-specific primers based on the plas-

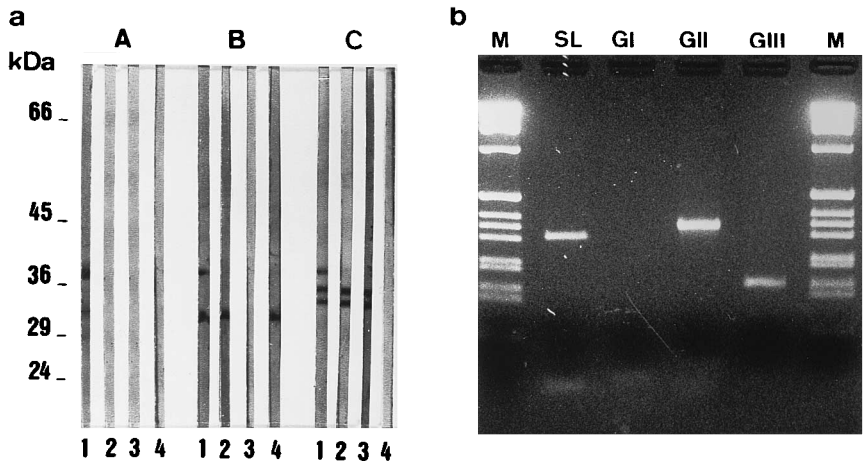

FIG. 4. (a) Western blot revealing specific epitopes of two *Borrelia* genospecies involved in Lyme disease. Samples of total proteins of strain B31, belonging to genospecies *B. burgdorferi* sensu stricto (panel A), N34, belonging to *B. garinii* (panel B), and UM01, belonging to *B. afzelii* (panel C) were subjected to SDS–10% PAGE gel electrophoresis and transferred to a PVDF membrane. These were incubated with the serum from a patient with meningoradiculitis (patient 3), absorbed with lysates of strain B31 (lanes 2), N34 (lanes 3), or UM01 (lanes 4) or not absorbed (lanes 1). Membrane-bound primary immunoglobulins were revealed by labelled secondary immunoglobulins (peroxidase-conjugated rabbit immunoglobulins to human IgA, IgG, and IgM; Dako P212). Molecular masses are indicated on the left. (b) Analysis of serum (from patient 3) by PCR with SL and the three genospecies-specific primers (GI, GII, and GIII) based on *ospA* gene sequences. A 10% portion of the reaction volume was analyzed on 2.0% agarose gel electrophoresis. Lanes M, 1-kb DNA ladder (Life Technologies).

TABLE 3. PCR and Western blot data obtained for human sera

Patient	*B. burgdorferi* sensu stricto		*B. garinii*		*B. afzelii*	
	PCR[a]	WB[b]	PCR	WB	PCR	WB
1	+	+	−	−	−	−
2	−	−	−	−	+	+
3	−	−	+	+	+	+
4	+	+	+	+	+	+
5	+	+	−	−	−	+
6	+	+	−	−	−	+

[a] PCR amplification with species-specific primers which differentiate the three major *B. burgdorferi* sensu lato genospecies. +, amplification; −, no amplification.

[b] WB, Western blot. The presence (+) or the absence (−) of specific epitopes is indicated (see text for details).

mid *ospA* sequence, we were able to differentiate the three genospecies associated with Lyme disease by PCR. The classification obtained by this method was in agreement with data obtained by other procedures, such as DNA-DNA hybridization, restriction fragment length polymorphism analysis, multilocus enzyme electrophoresis, and 16S rRNA signature nucleotide analysis (7, 22, 26, 27, 31–35, 37). In view of its sensitivity, specificity, and simplicity, the PCR approach based on genospecies-specific primers thus offers the possibility of investigating, with a large number of samples, the possible correlation between the clinical outcome of Lyme disease and the genotype of the infecting *B. burgdorferi* sensu lato strains.

In this study, the *B. burgdorferi* sensu lato- and genospecies-specific primers were used to analyze 18 cases of neuroborreliosis. The PCR data showed that most patients had been infected by *B. garinii* (Table 2), in agreement with previous observations showing the preferential association of this genospecies with neurological complications (1, 2). Moreover, PCR analysis revealed several cases of pluri-infection. This observation is physiologically relevant, since the amplifications were shown to be specific and not due to laboratory contamination.

On the other hand, an independent immunological approach, which identified genospecies-specific epitopes, confirmed the existence of pluri-infection in Lyme disease. In some cases (Table 3, patients 5 and 6), Western blot analysis revealed the presence of antibodies specific for one genospecies (*B. afzelii*), although the DNA was not amplified by PCR. This could be due to the absence of DNA or its presence at a level too low to be detected by PCR in the serum at the time of sampling. Alternatively, this species might have been eliminated from the patient, while the antibodies persisted in biological fluids. Furthermore, inconsistencies between the PCR and blotting data could result from sequence variation within the PCR target sites or from differences in distributions of spirochetal genospecies in host organs (23).

Altogether, the data obtained by the two approaches underscored the relatively high prevalence of pluri-infection in Lyme disease. Whether this phenomenon resulted from consecutive infections by distinct *B. burgdorferi* sensu lato genospecies, as reported before (25, 36), or was due to simultaneous multi-infection by a single tick, as described recently (17), remains to be established.

Finally, the sequences of amplified DNA from two cases were different from those of any of the isolated spirochetes included in this study. This stresses the possibility that *B. garinii* strains might be more heterologous than suspected up to now. This observation, together with that of multi-infection, might be of relevance in the evaluation of vaccines against *B. burgdorferi* sensu lato.

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